

Appl. #08/484,594

Filed: 6/7/95

By: J.S. O'Brien, et al. Group: 1818

Case Docket No.: MYELOS.002DV2

The Journal of Neuroscience, November 1995, 15(11): 7293-7300

Differential Susceptibility of Human CNS-Derived Cell Populations to TNF-Dependent and Independent Immune-Mediated Injury

Sameer D'Souza,¹ Karen Alinauskas,¹ Elille McCrea,¹ Cindi Goodyer,² and Jack P. Antel¹

¹Neuroimmunology Unit, Department of Neurology and Neurosurgery, and ²Department of Pediatrics, McGill University, Montreal, Quebec H3A 2B4, Canada

We examined whether oligodendrocytes, neurons, and astrocytes derived from the human central nervous system differ in susceptibility to injury mediated by tumor necrosis factor (TNF)- α and by activated CD4⁺ T cells acting via a TNF-independent mechanism. Injury was assessed either as cell membrane-directed (lysis), measured by ⁵¹chromium (Cr) or lactate dehydrogenase (LDH) release, or nucleus-directed (apoptosis), measured by morphologic features based on propidium iodide (PI) staining and by DNA fragmentation measured by a terminal transferase (TdT)-mediated dUTP biotin nick end labeling technique (TUNEL). TNF did not induce ⁵¹Cr or LDH release in any cell targets, but did induce nuclear (66 \pm 2% of cells) and DNA (68 \pm 2% of cells) fragmentation selectively in the oligodendrocytes over 96 hr. At this time, there was no significant loss of oligodendrocyte cell number. Nuclear injury could be induced in neurons by serum deprivation and in malignant astrocytes by the combination of TNF and low serum. CD4⁺ T cells activated with phytohemagglutinin (PHA) or anti-CD3 plus interleukin-2 induced significant ⁵¹Cr and LDH release in all target cells tested; only PHA-activated CD4⁺ T-cell cocultures showed reduced target cell numbers. Significant nuclear fragmentation was observed only for glioma cells (22 \pm 1% of cells). Differences in susceptibility to different immune effector mechanisms and in the nature of the injury response to the same effector mediator among human CNS-derived neural cells will need to be considered in design of therapeutic strategies aimed at protecting or limiting target cell injury consequent to disease or trauma.

[Key words: oligodendrocytes, cortical neurons, tumor necrosis factor α (TNF α), CD4⁺ T cells, cytotoxicity, immune injury of CNS]

Cell-mediated immune (CMI) responses are implicated as the primary mediators of tissue injury which occur in an array of inflammatory diseases of the CNS. Such responses can be mediated either by cell-cell contact-dependent or soluble factor-dependent mechanisms. In multiple sclerosis, myelin and its cell of origin, the oligodendrocyte (OL), are considered the major targets of this response (Bruck et al., 1994). Pathologic and neuroimaging studies indicate that axonal injury also occurs, partic-

ularly during the progressive phase of the disease (Arnold et al., 1994). In some viral diseases, including HIV encephalopathy, immune effectors contribute to the target cell injury (Wilt et al., 1995). Immune system products are also implicated as secondary contributors to the cellular injury in neurodegenerative disorders such as Alzheimer's disease (McGeer et al., 1989).

A specific issue raised with regard to immune mediators of tissue injury relates to whether different populations of CNS cells are selectively vulnerable to particular effectors. A corollary would be whether the mechanism of target cell injury to a common effector differs among neural cell populations. Cell injury has usually been considered in terms of primary cell membrane injury (lysis) or nuclear injury (apoptosis).

In the present study, we address these issues using dissociated *in vitro* cultures of human adult CNS-derived OLs, fetal CNS-derived neurons and astrocytes, and human glioma cell lines as target cells. As effector mediators, we used tumor necrosis factor (TNF)- α and *in vitro*-activated CD4⁺ T cells. The former, under pathologic conditions in the CNS, may be derived both from infiltrating mononuclear cells and glial cells, both microglia and astrocytes (Robbins et al., 1987; Lee et al., 1993; Williams et al., 1994). TNF α has been shown to induce DNA fragmentation of primary rodent, bovine, and human OLs after 72–96 hr of *in vitro* exposure (Selmaj et al., 1991) and of the OL precursor cell line CG4, induced to mature *in vitro* (Louis et al., 1993). The related molecule TNF β is at least as toxic to OLs (Selmaj et al., 1991). Gelbard et al. (1993) reported that TNF α was toxic to cultured primary human fetal cortical neurons, as assessed morphologically.

We have previously shown that mitogen- or antigen-activated CD4⁺ T cells, the T-cell subset used to transfer autoimmune encephalitis in animals, can induce OL cell membrane injury, measured using ⁵¹Cr or LDH release, within 18 hr of effector:target coculture (Antel et al., 1994). The effect is TNF-independent and non-MHC-restricted (Antel et al., 1994). At this time point, there is no evidence of nuclear injury, as measured by a DNA fragmentation assay (McLaurin et al., 1995). In the present study, we have assessed target cell responses to the T-cell effectors in terms of cell membrane and nucleus-directed injury over short (18 hr) and longer term (96 hr) culture. A non-MHC-restricted CD4⁺ T-cell effector response should be of particular interest in context of CNS cell injury, in that neither neurons or OLs to express MHC class II molecules, the restricting element for antigen-specific CD4⁺ T cells.

Materials and Methods

Preparation of effectors

Recombinant human TNF α used in these studies was obtained from Intermedico Diagnostics (Markham, Ontario). Preliminary studies es-

Received May 8, 1995; revised June 21, 1995; accepted July 5, 1995.

This work was supported by grants from the Medical Research Council of Canada (J.A.) and a Jeanne-Timmins Fellowship (S.D.S.).

Correspondence should be addressed to Dr. Jack P. Antel, Neuroimmunology Unit, Montreal Neurological Institute, 3801 University Street, Montreal, Quebec H3A 2B4, Canada.

Copyright © 1995 Society for Neuroscience 0270-6474/95/157293-08\$05.00/0

established 1000 units/ml as a standard dose to perform the comparative studies described in this report (D'Souza et al., unpublished observations).

CD4⁺ T cells were prepared from peripheral blood samples obtained from healthy volunteers using an initial Ficoll-hypaque (Pharmacia, Baie D'Urfe, Quebec) gradient centrifugation step (30 min at 500 × g) to obtain mononuclear cells (MNCs). T cells were isolated by a rosetting technique in which MNCs were incubated for 1 hr at 4°C with S-(2-aminoethyl) isothiuroniumbromide hydrobromide (AET) (Aldrich, Milwaukee, WI) treated sheep red blood cells and then centrifuged for 30 min on a Ficoll-hypaque gradient (Antel et al., 1994). The rosetted cells (T lymphocytes) were collected from the pellet and the sheep red blood cells were lysed by use of Gey's solution, a hypotonic lysis buffer, for 4 min at 4°C. The cells were then centrifuged for 30 min on a 43.5% Percoll (Pharmacia) gradient to remove large granular lymphocytes and incubated for 1 hr at 37°C with anti-CD8 monoclonal antibody (from ATCC hybridoma cell line CRL 8014) and complement (baby rabbit serum, Cedar Lane, Hamilton, Ontario). Following lysis, the remaining cells (CD4⁺ T cells) were collected, washed 3× in phosphate-buffered saline (PBS), and suspended at 1×10^6 cells per milliliter in culture medium comprised of RPMI (GIBCO, Burlington, Ontario) supplemented with 5% human serum (Pel Freeze, Brown Deer, WI), 2.5 µg per ml penicillin and 2.5 µg per ml streptomycin. Cells were placed in 25 cm² flasks (Falcon, Fisher Scientific, Montreal, Quebec) together with irradiated (2500 rads) autologous nonrosetting (E⁻) cells (2:1 ratio) as a source of feeder cells. Cells were activated *in vitro* for 72 hr with either soluble anti-CD3 antibody (10 µg per ml; ATCC CRL 8001), anti-CD3 plus recombinant human IL-2 (20 U/ml; gift of Immunex, Seattle, WA), or with phytohemagglutinin (PHA) (1 µg/ml; Wellmark Diagnostics, Guelph, Ontario). Cells were then harvested and centrifuged on a Ficoll-hypaque gradient to remove dead cells. Extent of T-cell activation was assessed by determining ³H-thymidine uptake over 5 hr by an aliquot of these cells. Purity of the CD4⁺ T cells was determined by FACS analysis. The enriched CD4⁺ T cells were washed twice and resuspended in RPMI plus 10% fetal calf serum (FCS). In some experiments, anti-CD3-stimulated cells were coated with PHA immediately prior to their use as effector cells.

Preparation of targets

Adult CNS-derived cells. OLs were derived from temporal lobe tissue specimens resected from young adults undergoing surgical resection for the treatment of intractable epilepsy. The method of cell isolation, previously described in detail (Yong and Antel, 1992) utilized an initial trypsinization step, followed by Percoll gradient centrifugation. Dissociated cells were cultured in minimum essential medium (MEM) supplemented with 5% fetal bovine serum (FBS), and 0.1% glucose. Subsequent separation of glial cell subtypes is based on their differential adhesion to uncoated Falcon tissue culture flasks (Fisher, Montreal, Canada) or petri dishes. Enriched populations of OLs were harvested from the mixed glial preparation in the form of the nonadherent cell fraction after 24 hr and replated on coated petri dishes. After a further 24 hr, the nonadherent cells were again collected and placed either into 96 well microtiter wells (5×10^4 cells per well) or onto poly-L-lysine-coated Aclar coverslips (5×10^4 cells per coverslip) which could be placed in 24 well microtiter wells. Cells were allowed to extend processes, being optimal for functional assays within 2–4 weeks from time of initial isolation.

Fetal CNS-derived cells. Neurons and astrocytes were derived from human fetal CNS tissue (cerebral hemispheres) obtained at 12–16 week gestation, using Medical Research Council of Canada approved guidelines. The cultures were prepared by dissociation of the fetal CNS tissue with 0.05% trypsin and 50 µg/ml DNase, passing the tissue through a 132 µm nylon mesh screen, and then through a 70 µm screen. After washing with PBS, the cells were suspended in culture medium (Eagle's MEM supplemented with 5% FBS, 0.1% glucose, and 1 mM sodium pyruvate), and then placed into poly-L-lysine-coated 96-well microtiter wells or onto Aclar coverslips which were then placed in petri dishes (5×10^4 cells per microwell or coverslip). To obtain neurons, culture dishes are first treated on day 4 *in vitro* with 1 mM 5-fluoro-2-deoxyuridine (5-FDU) for 2 d to deplete astrocytes; the treatment was repeated three times over a 2 week time period. These neurons are used in functional assays within 14 d.

To obtain astrocytes, nonreduced fetal CNS-derived dissociated cultures were passaged two or three times using 0.25% trypsin for 5 min at 37°C when cultures were confluent. Cells could be resceded

into either 96-well microwells or onto coverslips (2.5×10^4 cells per ml) 24 hr prior to use in functional assays.

Human glioma cell lines. Cell line U251 was obtained from ATCC, and maintained in serial passage in RPMI supplemented with 5% FCS. Cells were trypsinized and resceded for functional assays, as described for fetal astrocytes. Additional studies were conducted using the A172 glioma cell line; results were comparable to those with the U251 cells.

Immunocytochemistry

The individual neural cell types were characterized by immunostaining. OLs were identified using the mouse anti-R monoclonal antibody (mAb), H8H9 (Ranscht et al., 1982), derived from hybridoma supernatant, followed by goat anti-mouse IgG conjugated with Texas red (Jackson Immunoresearch Lab, West Grove, PA). Neurons were identified by staining with anti-MAP 2 mAb (1:1000 dilution), SMI52, (Sternberger-Meyer Immunocytochemicals, Whitehall, MD), followed by biotinylated horse anti-mouse IgG (1:100 dilution), followed by fluorescein-conjugated streptavidin (Boehringer-Mannheim, Laval, Quebec). Additional antibodies used to identify neurons included anti-neurofilament SMI33 (Sternberger-Meyer), anti-neuron-specific enolase (NSE) (Dako, Westchester, PA), and anti-Tau (Sigma #T-5530, St. Louis, MO) mAbs. Rabbit anti-glial fibrillary acidic protein (GFAP) Ab was used as the astrocyte marker. Neuron and astrocyte staining involved fixation of cells in acid alcohol (5% glacial acetic acid, 95% absolute alcohol) for 15 min. Except for fixation, which was done at -20°C, all steps were performed at room temperature. The OL-directed immunostaining was done on live cells without fixation. Cells on coverslips were incubated with all primary and secondary antibodies for 45 min; cells were washed three times in PBS between incubations. After staining, previously nonfixed cells were fixed in 2% paraformaldehyde (20 min).

Assays of neural cell injury

TNFα or CD4⁺ T cells were added to cultures of target cells contained in 24- or 96-well microtiter plates at concentrations of effector:target (E:T) ratios and for the time durations indicated in the Results section. Initial studies established that TNFα effects were concentration dependent and that CD4⁺ T-cell activity was dependent on E:T ratio (Antel et al., 1994). Most T-cell-mediated injury studies reported here were performed at a 10:1 E:T ratio.

Cell membrane-directed injury. These studies were conducted over either 5 or 18 hr using ⁵¹Chromium (Cr) release as the standard measure of cell membrane injury. For the ⁵¹Cr assay, target cells were labelled overnight with 0.1 µCi of ⁵¹Cr (NEN DuPont, Mississauga, Ontario) per well of 96-well microtiter plates. Cells were then washed twice in PBS before addition of TNFα or CD4⁺ T cells at indicated concentrations or E:T ratios. After 5 or 18 hr, 100 µl of cell-free supernatant was collected from individual microwells to determine induced ⁵¹Cr release (IR). One hundred microliters of 5 N sodium hydroxide (Fisher Scientific) was then added to each well for 30 min, and a further 100 µl was collected to determine residual ⁵¹Cr release (RR). Percentage non-specific ⁵¹Cr release (NSR) was determined from microwells containing only target cells by $(SR/(SR + RR)) \times 100$, where SR is spontaneous release. Percentage specific release was calculated as $[(IR/(IR + RR)) - NSR] \times 100$.

In some studies, an LDH release assay (as described below) was used, although in this assay the cell source (effector or target) of released LDH would be uncertain.

For the TNFα studies, the LDH release assay was used as the standard assay at both the 18 and 96 hr time points. For this assay, culture supernatants were collected and analyzed using a commercial kit (Sigma). Results are expressed as LDH units of test samples + LDH units of culture medium alone × 100%. The assay is insufficiently sensitive to detect LDH in serum-free conditions.

Nuclear injury (apoptosis). These studies were performed over a 18–96 hr time period on cells contained on coverslips placed into 24-well microtiter plates. Given that our cell targets of injury are nondividing primary cells and are available only in relatively small cell numbers, we have utilized, as our measures of nuclear injury, propidium iodide (PI) staining to identify changes in nuclear morphology (fragmentation, condensation of chromatin) and the terminal transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) technique to identify DNA fragmentation. PI staining was carried out on acetone:methanol or acid alcohol fixed coverslips. After rehydration for 30 min in PBS, coverslips

were stained with PI (10 μ g per ml, 20 min incubation), washed in PBS, and mounted for counting. For the TUNEL assay, coverslips containing the specific cell type were incubated for 1 hr at 37°C with 50 μ l of nick end labeling solution containing TdT (0.3 U/ml) and biotinylated dUTP (0.01 mmol/ml) in TdT buffer (Promega). The reaction was terminated by transferring the coverslips into microwells containing Tris buffer for 15 min at room temperature. After blocking with 2% BSA for 15 min and washing, the coverslips were incubated with streptavidin-FITC (1:20, 30 min at 37°C) (Boehringer-Mannheim).

We have used serum deprivation, a commonly used means to induce apoptosis in many cell lines, as a means to determine whether our target cells will show evidence of nuclear injury in the above assays.

TUNEL and PI quantitative data were obtained by counting coded slides. At least 200 cells per slide were counted. The number of coverslips counted and the number of individual experiments included for each test condition are indicated in the figures. In some studies, the total number of cells present in a defined area of a coverslip was determined under control and test conditions. ^{51}Cr and LDH data are presented as means \pm SEM for the number of experiments performed; each experiment was performed in triplicate.

Results

Properties of target cells

The morphologic and/or immunocytochemical features of the neural cells used in this study are illustrated in Figure 1. Purity of the OLs in enriched cultures was estimated at 80–90% during the 2–4 week culture period. Contaminating cells included astrocytes and fibroblasts. The latter overgrew the culture over longer time periods. The fetal cortical neurons were estimated to comprise 80–90% of cells in their cultures, with GFAP⁺ astrocytes being the other cell type. Although only immunoreactivity with MAP 2 mAb is illustrated, these cells could be immunostained with anti-neurofilament, anti-NSE, and anti-Tau mAbs. The U251 glioma line has lost GFAP immunoreactivity.

Target cell susceptibility to TNF α -mediated injury

Membrane-directed injury. Levels of LDH measured in supernatant of 5% serum-supplemented cultures of OLs, neurons, and astroglia did not differ between those which did or did not contain TNF α (Table 1).

Nucleus-directed injury. Addition of a single dose of 1000 units of TNF α to 5% serum-supplemented OL-enriched cultures resulted in nuclear fragmentation in a significant proportion of OLs at 96 hr ($66 \pm 3\%$, $n = 6$) compared to control cultures ($7 \pm 0.6\%$, $p < 0.01$) by PI criteria (Fig. 2). A similar proportion of OLs ($68 \pm 2\%$) showed DNA fragmentation as determined by TUNEL assay (Fig. 1, Table 2). This effect was not apparent at 24 hr. The proportion of OLs showing TNF α -induced nuclear fragmentation at 96 hr was significantly greater than that induced by serum deprivation ($43 \pm 3\%$, $n = 6$, $p < 0.01$). The number of OLs remaining in the TNF-treated and nontreated cultures at 96 hr did not differ significantly (Table 1).

The proportion of fragmented nuclei in neurons exposed to TNF for 96 hr was significantly increased over control values ($10 \pm 1\%$ vs $6 \pm 0.4\%$, $n = 7$, $p < 0.01$). This proportion was, however, markedly lower than that for OLs and significantly lower than that induced by serum deprivation ($25 \pm 3\%$, $n = 7$, $p < 0.001$).

The proportion of fragmented nuclei in glioma cells was not significantly increased in cultures containing 5% serum plus 1000 U/ml of TNF α ($7 \pm 0.9\%$, $n = 6$) compared to control values ($5 \pm 0.6\%$, $n = 6$) (Fig. 2). Serum deprivation alone augmented nuclear fragmentation only to a small extent ($12 \pm 2\%$, $n = 3$). Fragmentation of nuclei could be more readily observed in these cells by combining reduced serum conditions (1%) and TNF α ($35 \pm 3\%$, $n = 5$, $p < 0.001$). Similar results

were obtained with the TUNEL assay ($42 \pm 3\%$, $n = 3$) (Table 2). The fetal astrocytes did not undergo significant nuclear fragmentation (Fig. 2) or DNA fragmentation in response to either TNF α and/or low serum conditions. The overall correlation coefficient between the results of the PI assay of nuclear fragmentation and the TUNEL assay of DNA fragmentation in the above TNF assays and the CD4⁺ T-cell effector assays described below was 0.979, $n = 14$.

Target cell susceptibility to CD4⁺ T-cell-mediated injury

Membrane-directed injury. As shown in Figure 3 and Table 1, the membrane-directed lytic capacity of CD4⁺ T cell at 24 hr was found to be dependent on the method of T-cell activation. PHA-activated CD4⁺ T cells induced significant ^{51}Cr release from each of the neural cell targets, with release being highest in the proliferating cell targets (fetal astrocytes and glioma cells). These results were confirmed in the LDH assay (Table 1). Significant loss of both OLs and glioma cells, the only two target cells examined, was observed following their exposure to the PHA-activated CD4⁺ T cells (Table 1). PHA-activated CD4⁺ T-cell-mediated ^{51}Cr release could be detected as early as 5 hr in effector:target cocultures (data not shown).

CD4⁺ T cells activated with anti-CD3 and IL-2 also induced significant ^{51}Cr release from all neural cell targets tested in the 18 hr assay, with no apparent differences amongst the targets in their relative susceptibilities (Fig. 3). Significant cell loss could not be detected for either the OLs or gliomas (Table 2). CD4⁺ T cells activated by anti-CD3 alone did not induce significant ^{51}Cr release from neurons, astrocytes, or glioma cells (Fig. 3). A low and variable level of OL-directed cytotoxicity was found. Studies using glioma cells as targets indicated that anti-CD3-activated T cells could induce ^{51}Cr release of previously resistant targets if the effector cells were coated with PHA just prior to being added to the previously "resistant" target cells ($6 \pm 5\%$ for uncoated vs $19 \pm 2\%$ for PHA-coated anti-CD3-activated effectors, $n = 2$).

^3H -thymidine uptake was comparable between CD4⁺ T cells activated by PHA (34226 ± 5591 cpm, $n = 4$) or anti-CD3 (38355 cpm \pm 5534, $n = 4$); uptake by anti-CD3 and IL-2-activated cells was significantly higher (59252 ± 7788 cpm, $n = 4$, $p < 0.05$). Purity of CD4⁺ T cells did not differ as a function of activation conditions (mean percent CD4⁺ T cells derived from anti-CD3-activated cultures was $89 \pm 3\%$; from anti-CD3 plus IL-2-activated cultures $87 \pm 3\%$; and, from PHA-activated cultures $92 \pm 2\%$, $n = 7$) as assessed using flow cytometry. There were less than 2% CD8⁺ T cells or NK cells.

Total ^{51}Cr labelling of the various cell targets used did not vary widely (for OLs: 5000–7000 cpm; for neurons: 2500–3000 cpm; for astrocytes and glioma cells: 6000–8000 cpm). Non-specific release was comparable among the neural cells, with a mean range of 26–33%. These values are higher than those found using a 5 hr ^{51}Cr release assay.

Nucleus-directed injury. Although all target cells released ^{51}Cr upon coculture with PHA-activated CD4⁺ T cells, only in the glioma cells was there a significant increase in the proportion of fragmented nuclei compared to control cultures, as measured either by PI staining or TUNEL technique (Fig. 2, Table 2). The proportion was increased as early as 18 hr, with a further increase by 96 hr.

Discussion

We have utilized dissociated cultures of human CNS-derived cells to examine the relative susceptibility of various

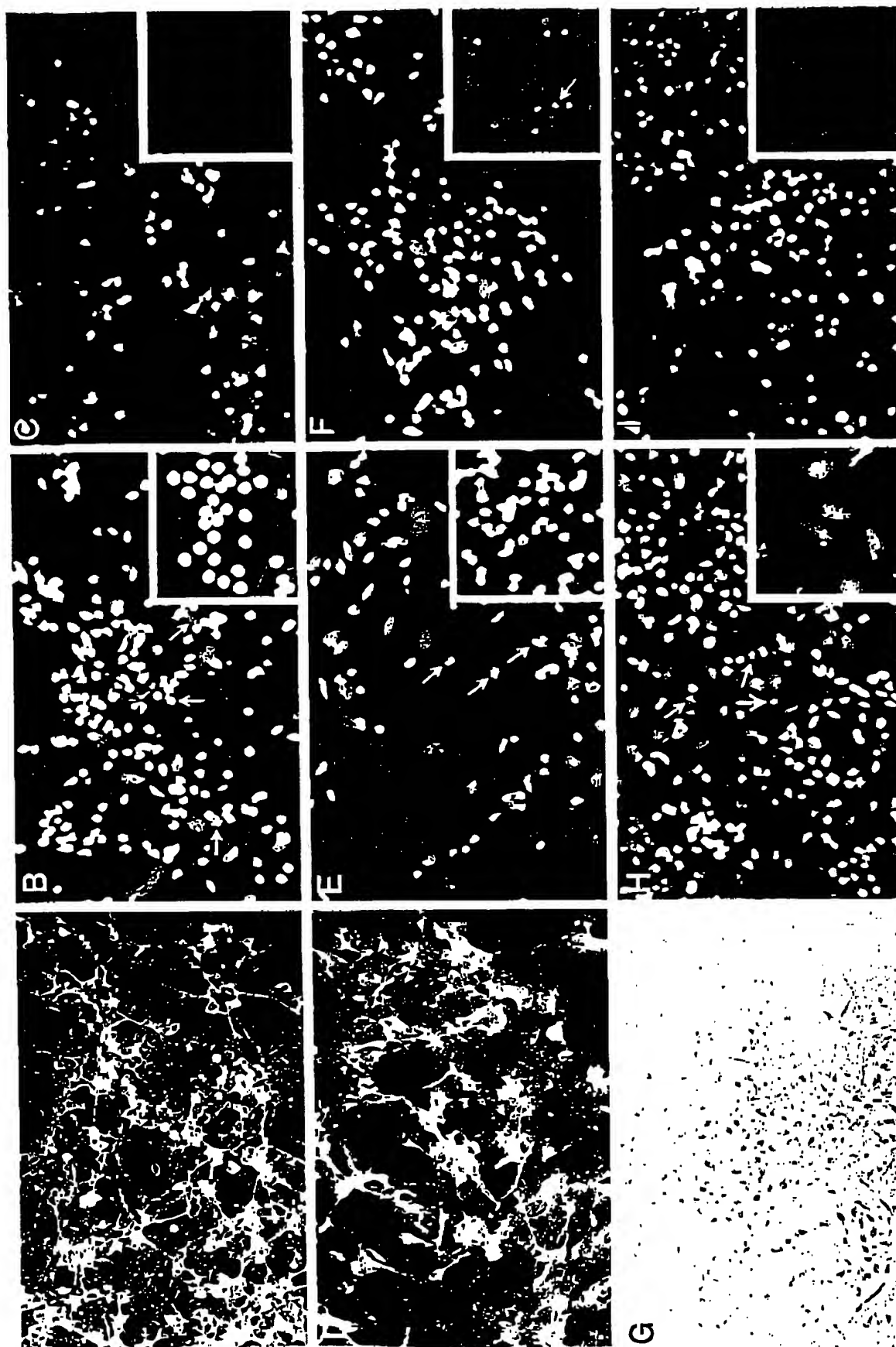


Figure 1. Induction of DNA fragmentation in human neural cells: *A*, adult CNS-derived oligodendrocytes (OLs) maintained in 5% serum-supplemented culture medium (control culture) and immunostained with anti-R mAb (350X); *B*, OLs cultured in 5% serum-supplemented medium plus 1000 U/ml TNF α and stained with PI (450X); arrows indicate examples of fragmented or condensed (apoptotic) nuclei; *C*, OLs cultured as in *B* and stained with TUNEL technique to demonstrate DNA fragmentation (350X). Insets in *B* and *C* indicate OLs grown in control medium and stained with PI and TUNEL (450X and 350X, respectively). *D*, Human fetal CNS-derived cortical neurons maintained in control medium and immunostained with anti-MAP2 Ab (350X); *E*, neurons cultured in serum-depleted medium and stained with PI and TUNEL (450X and 350X, respectively). Arrows in *E* and *F* indicate apoptotic nuclei (450X); *F*, neurons cultured as in *E* and stained with TUNEL technique (350X). Insets in *F* indicate apoptotic nuclei (450X). *G*, phase contrast micrograph of GFAP $^{+}$ human glioma line U251 maintained in control medium (350X); *H*, glioma cells maintained in 1% serum and 1000 U/ml TNF α and stained with PI and TUNEL (350X); *I*, glioma cells cultured as in *H* and stained with TUNEL technique (350X). Arrows in *H* and *I* are control cultures stained with PI and TUNEL.

BEST AVAILABLE COPY

Table 1. Effects of TNF α and activated CD4 $^{+}$ T cells on target cells—release of LDH and cell survival

Effector	Target cells			Surviving cell numbers (% of control)	
	LDH release (% of control)				
	OLs	Neurons	Glioma	OLs	Glioma
TNF α	96 \pm 1% <i>n</i> = 3	121 \pm 11% <i>n</i> = 3	110 \pm 6% <i>n</i> = 3	102 \pm 12% <i>n</i> = 4	ND
CD4 $^{+}$ T cells + pha	173% <i>n</i> = 3	ND	250% (249,250) <i>n</i> = 3	40 \pm 9% <i>n</i> = 3	29 \pm 4% <i>n</i> = 3
anti-CD3-IL2	185% <i>n</i> = 3	ND	206% (186,225) <i>n</i> = 3	103 \pm 30% <i>n</i> = 3	97 \pm 7% <i>n</i> = 3
anti-CD3	126% <i>n</i> = 2	ND	126% (135,117) <i>n</i> = 3	92 \pm 19% <i>n</i> = 3	91 \pm 2% <i>n</i> = 3

Comparison of LDH release and number of surviving target cells in effector:target cocultures with that found in cultures containing target cells alone. For LDH studies, data are expressed as LDH in test cultures \div LDH in control cultures \times 100% for the number of experiments performed \pm SEM, except when only single experiments (single value shown) or duplicate experiments (averaged and individual results shown) were performed. For single and duplicate experiments, each data point represents mean of triplicate cultures. Culture medium alone contains 400–600 U of LDH. LDH values of cultures containing target cells only did not differ significantly from medium alone values. For cell survival studies, data are expressed as number of target cells in a defined coverslip region of an effector:target coculture \div number of cells in a defined coverslip region of a target-cell-only culture \times 100% \pm SEM for the number of experiments performed. ND, Not done.

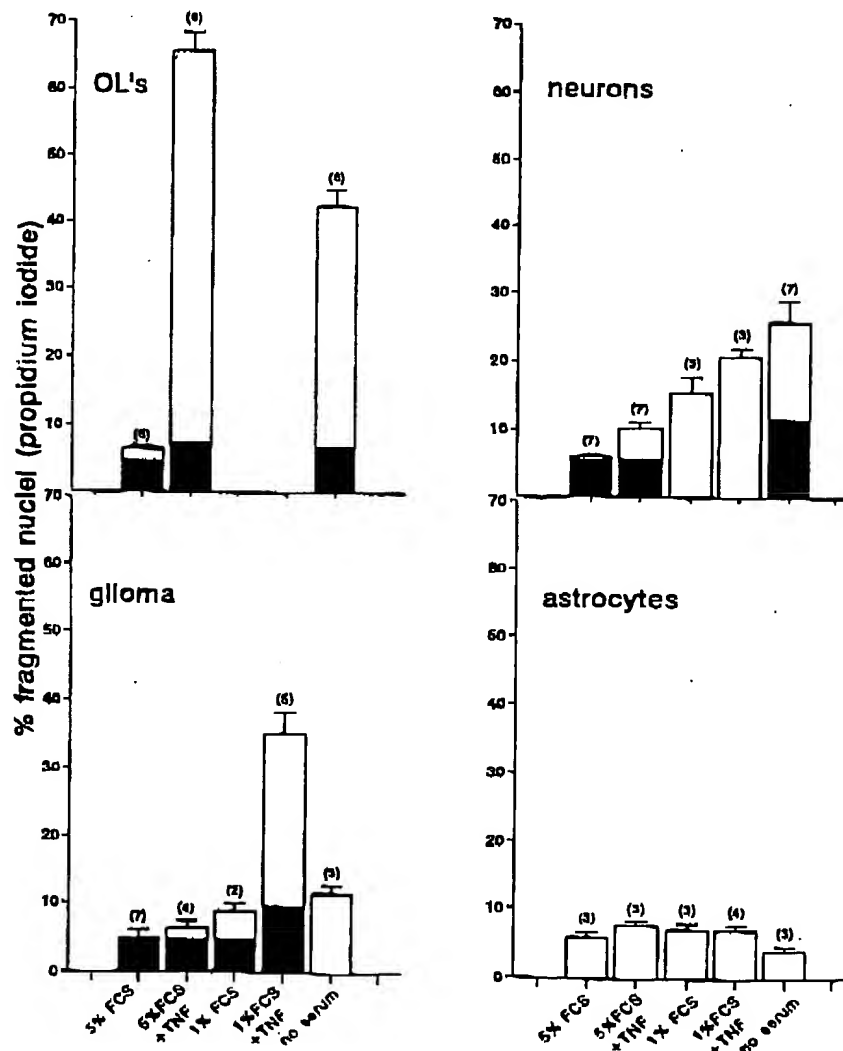


Figure 2. Susceptibility of human neural cells to TNF α -induced nuclear injury, propidium iodide (PI) assay; bars indicate percentage \pm SEM of OLs, neurons, glioma, and fetal astrocytes which show morphologic features of nuclear fragmentation after 96 hr in tissue culture medium containing either 5% FCS, 5% FCS plus 1000 U/ml TNF α , 1% FCS, 1% FCS plus TNF α , or no serum. Solid portions of bars indicate percentage of cells with fragmented nuclei found at 18 hr. For each test condition the number of coverslips counted is indicated. At least two individual experiments were performed for each condition.

Table 2. Extent of DNA fragmentation (TUNEL assay) of target cells in response to effector conditions inducing significant nuclear fragmentation (PI assay)

Target cell	Effector condition	TUNEL (% positive cells)	PI (% fragmented nuclei)
OLs	TNF α	68 \pm 2% (6)	66 \pm 3% (6)
	Serum deprivation	ND	43 \pm 3% (6)
Neurons	Serum deprivation	32 \pm 2% (2)	25 \pm 3% (7)
Glioma	TNF α + 1% serum	42 \pm 3% (3)	35 \pm 3% (5)
	pha-activated CD4 $^{+}$ T cells	33	22 \pm 1% (4)

Data indicate results of PI and TUNEL assays obtained for the same test conditions, in which significantly elevated values for the proportion of cells with nuclear injury were found. Data are expressed as mean \pm SEM% for the number of coverslips counted. At least two experiments were done for each condition, except glioma plus pha-activated T cells where a single coverslip was counted.

and non-TNF-dependent effector mechanisms, the latter mediated by activated CD4 $^{+}$ T cells acting in a non-MHC-restricted manner. The target response was assessed in terms of the two major categories of cell injury, namely primary cell membrane-directed injury (lysis) and nucleus-directed injury (apoptosis) (Zychlinsky et al., 1991; Taylor and Cohen, 1992; Cohen, 1993; Louis et al., 1993; Schwartzman and Codlowski, 1993), and in

terms of the time required for the response to develop. The results indicated that the target cells studied (OLs, neurons, and malignant and nonmalignant astrocytes) differed in their relative susceptibilities to these effector mechanisms and in the nature of the cellular injury which occurred.

The susceptibility of human adult CNS-derived OLs to the nuclear injury-inducing effects of TNF α that we found in our

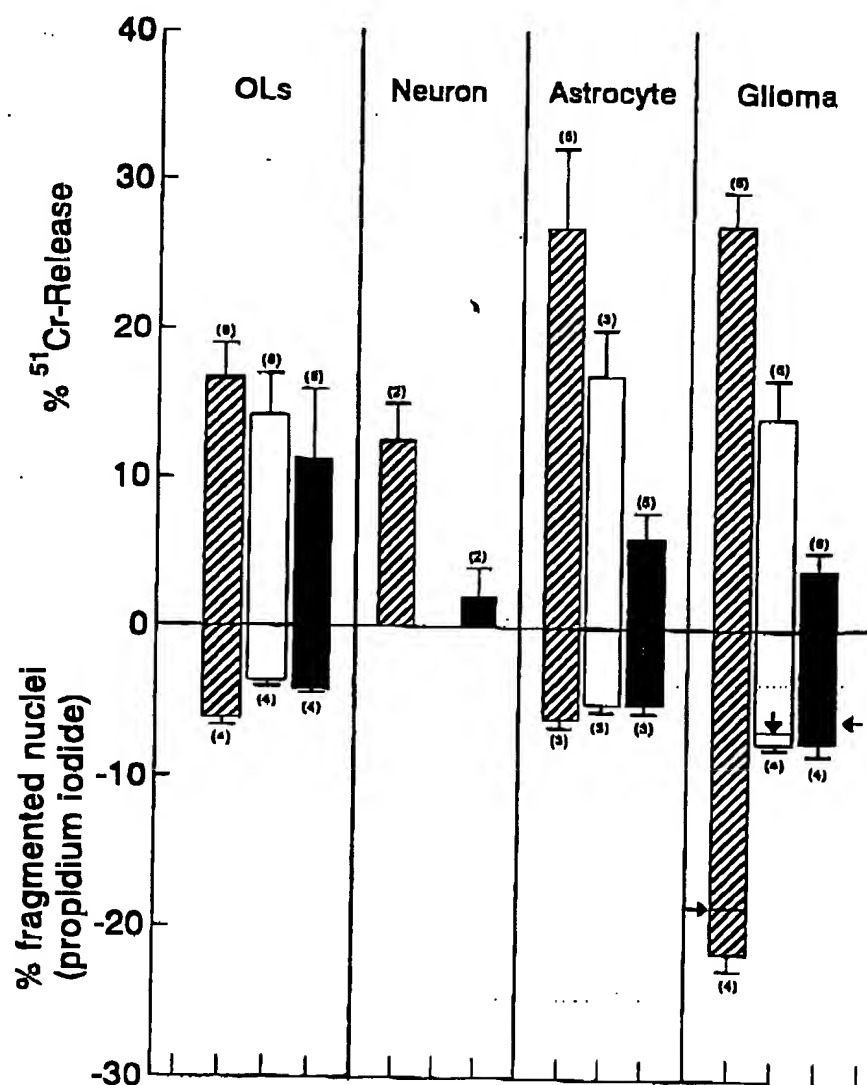


Figure 3. Susceptibility of human neural cells to cell membrane injury as measured by ^{51}Cr release and to nuclear fragmentation as measured by PI staining, induced by CD4 $^{+}$ T cells activated by either PHA antibody (hatched bars), anti-CD3 plus IL-2 (open bars), or anti-CD3 antibody (solid bars). Studies were performed at 10:1 E:T ratios. Upper panel indicates mean percentage specific ^{51}Cr release \pm SEM at 18 hr for the number of individual studies indicated for each target cell type. Lower panel indicates mean percentage \pm SEM of fragmented nuclei for the corresponding target cells, as determined at 96 hr; 18 hr data is indicated by arrows. Number of coverslips or experiments used in each condition is indicated.

study are consistent with those observed by others using rodent and bovine primary OL cultures and a rodent OL-precursor cell line (Sclmaj et al., 1991; Louis et al., 1993). Over the same time period, we did not detect significant cell membrane rupture, as assessed using ^{51}Cr or LDH release, nor loss of cell numbers. The latter results are consistent with those of Zajicek et al. (1992), who found that soluble human recombinant TNF α did not induce cytotoxicity of rodent OLs, whereas cell-bound TNF was the putative mediator of microglia-induced cytotoxicity of OLs *in vitro*. In contrast to the OLs, the neurons, in our study, were relatively resistant to TNF α in terms of nuclear injury. The extent of TNF α -induced nuclear injury of OLs was greater than that induced by serum deprivation; the reverse was observed for the human fetal neurons. Giulian et al. (1993) previously observed the greater susceptibility of neurons, compared to OLs, to noncytokine low molecular weight factors released by microglia.

Neither the malignant nor nonmalignant fetal astrocytes showed significant nuclear injury in response to TNF α under our usual culture conditions. In addition, we did not observe significant nuclear or DNA fragmentation in the small proportion of astrocytes contained in our adult CNS-derived OL-enriched cultures. These results indicate the differences in susceptibility to a common effector between glial cells of apparently shared developmental lineage (Barres et al., 1992). The glioma cells, but not the nonmalignant astrocytes, showed significant nuclear and DNA fragmentation in response to the combination of serum deprivation and TNF α . The glioma cells had a higher rate of proliferation than did the fetal astrocytes; the proliferation of the latter was higher than that of the adult CNS-derived astrocytes (Yong et al., 1992). The adult astrocytes can be shown to incorporate significant levels of BrdU *in vitro* in contrast to the OLs (Grenier et al., 1989).

Nuclear injury was assessed using morphologic criteria of PI-stained cells and a fluorescence DNA fragmentation assay (TUNEL). A strong correlation was found between results in the two assays. The numbers of OLs and neurons available and difficulties in recovering these cells from cultures limited the use of other techniques to assess DNA fragmentation, such as DNA ladders or FACS analysis. The former would have limited use in the coculture systems discussed below, containing both effector and target cells.

Target cell injury mediated by CD4 $^{+}$ T cells depended both on the means of T-cell activation and the cell type serving as the target. Pha-activated CD4 $^{+}$ T-cell lysis could be detected in 5 hr as well as 18 hr cocultures, suggesting that these cells had residual surface lectin, and their effect was akin to lectin-dependent cell cytotoxicity (Bevan and Cohen, 1975; Ruijs et al., 1990). Our previous studies indicated that the effect was not mediated via TNF-dependent mechanisms, in that it could not be blocked with neutralizing anti-TNF antibody or reproduced with TNF (Antel et al., 1994). CD4 $^{+}$ T cells have previously been demonstrated to be capable of mediating both TNF-dependent and -independent cytotoxicity (Ju et al., 1990; Liu et al., 1992; Smyth et al., 1992).

CD4 $^{+}$ T cells activated by anti-CD3 and IL-2 induce membrane injury to a greater extent in the 18 hr than in the 5 hr cytotoxicity assays (Ruijs et al., 1990). The cytotoxic effect of anti-CD3 plus IL-2-activated CD4 $^{+}$ T cells would seem akin to that initially termed promiscuous killing in assay systems using either antigen or anti-CD3 and IL-2 to generate T-cell lines as effectors and dividing non-neural NK cell-resistant tumor lines

as targets (Patel et al., 1987; Thiele and Lipsky, 1989). The CD4 $^{+}$ T cells activated by anti-CD3 alone did possess cytotoxic potential, as revealed by coating these cells with pha immediately prior to adding them to target cells. IL-2 augmented cytotoxicity mediated by anti-CD3-activated T cells has previously been described (Stohl et al., 1990; Nishimura et al., 1992). The lack of cytotoxicity found using CD4 $^{+}$ T cells activated with anti-CD3 alone, compared to anti-CD3 plus IL-2, suggests that residual anti-CD3 antibody on the T cells is not serving as a ligand. The essential ligands involved in promiscuous T-cell killing of particular cell targets remains to be defined.

Extent of T-cell proliferation as a function of method of T-cell activation did not correlate with cytotoxicity, in that ^3H -thymidine uptake was similar between anti-CD3- and pha-activated T cells. T cells activated with anti-CD3 and IL-2 showed the highest proliferation rates. However, even at a 20:1 E:T ratio, CD4 $^{+}$ T cells activated by anti-CD3 alone did not induce the extent of ^{51}Cr release found using anti-CD3 plus IL-2-activated CD4 $^{+}$ T cells at a lower E:T ratio (data not shown), suggesting that differences in effector cell numbers that may have occurred during the co-culture period did not account for the functional results. Any remaining exogenous IL-2 in the anti-CD3 plus IL-2-activated cultures would have been removed at the time the T cells were washed prior to adding them to the cell targets. The T-cell populations used were highly enriched for CD4 $^{+}$ T cells, and thus the effects induced are unlikely to be due to a small population of NK cells (Galandrini et al., 1994). Mechanisms of CD4 $^{+}$ α/β T-cell receptor (TcR) bearing T-cell-mediated cytotoxicity may differ from those used by other T-cell populations, including CD8 $^{+}$ T cells and γ/δ TcR-bearing T cells (Smyth, 1992).

With regard to the target cell response to pha-activated T cells, only in the glioma cells did DNA and nuclear fragmentation develop. Significant fragmentation was observed within 18 hr, a time period which precedes that required for TNF α and serum depletion-induced fragmentation to occur. Cell numbers were reduced in all cell cultures exposed to pha-treated CD4 $^{+}$ T cells. The lack of DNA and nuclear fragmentation in the nonglioma neural cells would seem to differ from previous studies of CD4 $^{+}$ T-cell-mediated cytotoxicity using non-neural cell targets, in that the latter suggested that nuclear injury preceded cell disintegration (reviewed in Thiele and Lipsky, 1989; Duke, 1992; Grogg et al., 1992; Berke, 1994; Stalder et al., 1994). The glioma cells are the most actively proliferating of the cell targets used in our study. Our data suggests that primary non-dividing CNS cells, OLs, and neurons, are resistant to this pathway, although they are capable of undergoing apoptosis as shown using either TNF or serum deprivation. These data would be consistent with the existence of multiple different pathways whereby nuclear injury programs are activated (Rose and Henneberry, 1993; Schwartz et al., 1993; Schwartzman and Cidlowski, 1993; Oltavi and Korsmeyer, 1994).

Cell membrane injury mediated by the anti-CD3-activated CD4 $^{+}$ T cells was not accompanied by either the extent of DNA fragmentation (in the case of gliomas) or cell loss (found with all cell targets tested) observed using the pha-activated T cells. These findings could provide a model whereby T cells could induce membrane injury without necessarily cell body loss, that is, sublethal injury with potential for recovery, as suggested for complement-mediated injury (Scolding et al., 1989). Our previous studies did indicate that human OLs are susceptible to promiscuous cell-mediated cytotoxicity mediated by MRP...

active CD4⁺ T-cell lines (Antel et al., 1994). In the case of multiple sclerosis, the issue of myelin loss, with or without OL cell loss, continues to be studied (Bruck et al., 1994).

In summary, our current *in vitro* data indicate the differing susceptibility and cellular response of human CNS neural cells to TNF-dependent and independent immune effector mechanisms. Therapeutic strategies applied to diseases or injury of the human CNS will need to consider this heterogeneity.

References

- Antel JP, Williams K, Blain M, McCrea E, McLaurin J (1994) Oligodendrocyte lysis by CD4⁺T cells independent of tumor necrosis factor. *Ann Neurol* 35:341-348.
- Arnold DL, Riess GT, Matthews PM, Francis G, Collins DL, Wolfson C, Antel JP (1994) Use of proton magnetic resonance spectroscopy for monitoring disease progression in multiple sclerosis. *Ann Neurol* 36:76-82.
- Barras BA, Hart IK, Coles HSR, Burne JF, Voyvodic JT, Richardson WD, Raff MC (1992) Cell death and control of cell survival in the oligodendrocyte lineage. *Cell* 70:31-346.
- Berke G (1994) The binding and lysis of target cells by cytotoxic lymphocytes: molecular and cellular aspects. *Annu Rev Immunol* 12:735-773.
- Bevan MH, Cohen M (1975) Cytotoxic effects of antigen- and mitogen-induced T cells on various targets. *J Immunol* 114:59-565.
- Bruck W, Schmied M, Suchanek G, Cruck Y, Breitschopf H, Poser S, Piddlesen S, Lassmann H (1994) Oligodendrocytes in the early course of multiple sclerosis. *Ann Neurol* 35:65-73.
- Cohen JJ (1993) Apoptosis. *Immunol Today* 14:126-130.
- Duke RC (1992) Apoptosis in cytotoxic T lymphocytes and their targets. *Semin Immunol* 4:497-512.
- Galandrini R, De Maria R, Piccoli M, Frati L, Santoni A (1994) CD4⁺ triggering enhances human NK cell cytotoxic functions. *J Immunol* 153:4399-4407.
- Gelbard HA, Dzenko KA, DiLoreto D, Delcerro C, Delcerro M, Epstein LG (1993) Neurotoxic effects of TNF α in primary human neuronal cultures are mediated by activation of the glutamate AMPA receptor subtype—implications for AIDS neuropathogenesis. *Dev Neurosci* 15:417-442.
- Giulian D, Vaca K, Corpuz M (1993) Brain glia release factors with opposing actions upon neuronal survival. *J Neurosci* 13:229-237.
- Grenier Y, Ruijs TCG, Robitaille Y, Olivier A, Antel JP (1989) DNA synthesis by young adult human-derived astrocytes *in vitro*. *Brain Res* 480:87-91.
- Grogg D, Hahn S, Erb P (1992) CD4⁺ T cell-mediated killing of major histocompatibility complex class II-positive antigen-presenting cells (APC). III. CD4⁺ cytotoxic T cells induce apoptosis of APC. *Eur J Immunol* 22:267-272.
- Ju S-T, Ruddle NH, Strack P, Dorf ME, DeKruyff RH (1990) Expression of two distinct cytolytic mechanisms among murine CD4 subsets. *J Immunol* 144:23-31.
- Lee SC, Liu W, Dickson DW, Bronsman CF, Berman JW (1993) Cytokine production by human fetal microglia and astrocytes. Differential induction by lipopolysaccharide and IL-1 beta. *J Immunol* 150:2659-2667.
- Louis JC, Magal E, Takayama S, Varon S (1993) CNTF protection of oligodendrocytes against natural and tumor necrosis factor-induced death. *Science* 259:689-692.
- McGeer PL, Akiyama H, Itagaki S, McGeer EG (1989) Immune system response in Alzheimer's disease. *Can J Neurol Sci* 16:516-527.
- McLaurin J, D'Souza S, Stewart J, Blain M, Beaudet A, Nalbantoglu J, Antel JP (1995) Effect of tumor necrosis factor α and β on human oligodendrocytes and neurons in culture. *Int J Dev Neurosci* 13:369-381.
- Nishimura T, Nakamura Y, Takeuchi Y, Tokuda Y, Iwasawa M, Kawasaki A, Okumura T, Haba S (1992) Generation, propagation, and targeting of human CD4⁺ helper/killer T cells induced by anti-CD3 monoclonal antibody plus recombinant IL-2. *J Immunol* 148:285-291.
- Otavi AN, Korsmeyer SJ (1994) Checkpoints of dueling dimers foil death wishes. *Cell* 79:189-192.
- Patel SS, Thiele DL, Lipsky PE (1987) Major histocompatibility complex—unrestricted cytolytic activity of human T cells. Analysis of precursor frequency and effector phenotype. *J Immunol* 139:3886-3895.
- Prabakhar S, D'Souza SD, Antel JP, McLaurin J, Schipper HM, Wang E (1995) Phenotypic and cell cycle properties of adult human oligodendrocytes *in vitro*. *Brain Res* 672:159-169.
- Rauscht B, Clapshaw PA, Price J, Noble M, Seifert W (1982) Development of oligodendrocytes and Schwann cells studied with a monoclonal antibody against galactocerebroside. *Proc Natl Acad Sci USA* 79:2709-2713.
- Robbins DS, Shirazi Y, Drysdale B-E, Lieberman A, Shin HS, Shin MS (1987) Production of cytotoxic factor for oligodendrocytes by stimulated astrocytes. *J Immunol* 139:2593-2597.
- Rose CD, Henneberry RC (1993) Mechanisms of programmed cell death and their implications for the brain. *Neurodegeneration* 2:287-298.
- Ruijs TCG, Freedman MS, Grenier YG, Olivier A, Antel JP (1990) Human oligodendrocytes are susceptible to cytotoxicity by major histocompatibility complex class I-restricted lymphocytes. *J Neuroimmunol* 27:88-97.
- Schwartz LM, Smith SW, Jones MEE, Osborne BA (1993) Do all programmed cell deaths occur via apoptosis? *Proc Natl Acad Sci USA* 90:980-984.
- Schwartzman RA, Cidlowski JA (1993) Apoptosis: the biochemistry and molecular biology of programmed cell death. *Endocrine Rev* 14:133-151.
- Scolding NJ, Morgan BP, Houston WJ, Linington C, Campbell AK, Compston DAS (1989) Vesicular removal by oligodendrocytes of membrane attack complexes formed by activated complement. *Nature* 339:620-622.
- Selmaj K, Raine CS, Farooq M, Norton WT, Brosnan CF (1991) Cytotoxicity against oligodendrocytes. Apoptosis induced by lymphotoxin. *J Immunol* 147:1522-1529.
- Smyth MJ (1992) Generation and cytotoxic profile of human peripheral blood CD4⁺ T lymphocytes. *Immunol Cell Biol* 70:379-380.
- Smyth MJ, Norishia Y, Ortaldo JR (1992) Multiple cytolytic mechanisms displayed by activated human peripheral blood T cell subsets. *J Immunol* 148:55-62.
- Stalder T, Hahn S, Erb P (1994) Fas antigen is the major target molecule for CD4⁺ T cell-mediated cytotoxicity. *J Immunol* 152:1127-1133.
- Stohl W, Tovar Z, Talal N (1990) Generation of cytolytic activity with anti-CD3 monoclonal antibodies involves both IL-2-independent and -dependent components. *J Immunol* 144:3718-3725.
- Taylor MK, Cohen JJ (1992) Cell-mediated cytotoxicity. *Curr Opin Immunol* 4:338-343.
- Thiele DL, Lipsky PE (1989) The role of cell surface recognition structures in the initiation of MHC-unrestricted "promiscuous" killing by T cells. *Immunol Today* 10:375-381.
- Williams K, Dooley N, Ulvestad E, Blain M, Yong VW, Antel JP (1994) Antigen presentation by astrocytes: correction of the inability of astrocytes to initiate immune responses by the addition of microglia or the microglia-derived cytokine IL-1. *J Neurosci Res* 38:433-443.
- Wilt SG, Milward E, Zhou JM, Nagasato K, Patton H, Rusten B, Griffith D, O'Connor M, Dubois-Dalcq M (1995) *In vitro* evidence for a dual role of tumor necrosis factor- α in human immunodeficiency virus type 1 encephalopathy. *Ann Neurol* 37:381-394.
- Yong VW, Antel JP (1992) Culture of glial cells from human brain biopsies. In: *Protocols for neural cell culture* (Fedoroff S, Richardson A, eds), pp 81-96. New York: Humana.
- Yong VW, Tejada-Berges T, Goodyer C, Antel JP, Yong FP (1992) Differential proliferative response of human and mouse astrocytes to gamma-interferon. *Glia* 6:269-280.
- Zajicek JP, Wing M, Scolding NJ, Compston DAS (1992) Interactions between oligodendrocytes and microglia. A major role for complement and tumor necrosis factor in oligodendrocyte adherence and killing. *Brain* 115:1611-1631.
- Zychlinsky A, Zhen LM, Liu C-C, Yong JD-E (1991) Cytolytic lymphocytes induce both apoptosis and necrosis in target cells. *J Immunol* 146:393-400.